PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 5/10, A61K 48/00, 35/36

A1

(11) International Publication Number: WO 96/09373

(43) International Publication Date: 28 March 1996 (28.03.96)

(21) International Application Number: PCT/GB95/02187

(22) International Filing Date: 15 September 1995 (15.09.95)

(30) Priority Data:
9419048.5 20 September 1994 (20.09.94) GB

(71) Applicant *(for all designated States except US)*: BRITISH TECHNOLOGY GROUP LIMITED [GB/GB]; 101 Newington Causeway, London SE1 6BU (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WATT, Diana, Joan [GB/GB]; Woolmers, Brighton Road, Mannings Heath, West Sussex RH13 6HZ (GB). BETTENCOURT DE MEDEIROS RELVAS, Joao, Carlos [PT/PT]; Rua Machado de Castro, 22 Rc Esq A, P-3000 Coimbra (PT).

(74) Agent: PERCY, Richard, Keith; British Technology Group Limited, Patents Dept., 101 Newington Causeway, London DE1 6BU (GB).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: FIBROBLASTS FOR THE TREATMENT OF MUSCULAR DISORDERS

(57) Abstract

Muscular disorders such as Duchenne muscular dystrophy are alleviated by introducing dermal fibroblasts (which may be the patient's own), into the muscle. *In vivo* fusion of the donor cells with each other converts them into muscle cells, which produce the products of muscle-specific genes such as dystrophin and other muscle proteins. The donor fibroblasts may contain muscle-specific DNA.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

Fibroblasts for the treatment of muscular disorders

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

This invention relates to the treatment of muscular disorders, in particular those resulting from the wastage of muscle such as muscular dystrophy or from muscle degeneration, e.g. as a result of traumatic injury.

2. DESCRIPTION OF THE RELATED ART

Skeletal muscle cells are composed of multinucleated cylindrical structures 10-100 μm in diameter and of lengths of the order of millimetres or even centimetres, surrounded by a plasma membrane. The cells are capable of contraction and arise by fusion of myoblasts, which are immature muscle cells.

One of the commonest forms of muscle disease is Duchenne muscular dystrophy (DMD). It is caused by an X-chromosome-linked genetic defect which results in a lack of production of the protein dystrophin. Since it is unlikely that the defect will occur in linkages to both X chromosomes of a female, and since it is recessive in character, it is transmitted only to males through the defective X-linkage. Becker muscular dystrophy (BMD) also results from an X-linked genetic defect.

Following the cloning of the DMD gene, interest has heightened in using a form of therapy, termed myoblast transfer therapy, to alleviate the myopathic condition in DMD muscles. Thus, J. E. Morgan *et al.*, J. Cell Biology *111*, 2437-2449 (Dec 1990) worked with irradiated muscular dystrophic (*mdx*) mice. *Mdx* mice, unlike humans, spontaneously regenerate the affected muscle. Irradiation of an area of the skeletal muscle makes it unable to regenerate the muscle in this area. These authors showed that muscle degenerated by dystrophin deficiency in the skeletal muscle of *mdx* mice can be regenerated by implanting neonatal muscle precursor cells (myoblasts) from wild-type (i.e. non-diseased) mice. These authors inhibited the natural regeneration of muscle in *mdx* mice by applying a high dose of X-rays locally to a leg of the mice 3 days before injecting them with the culture of the neonatal cells. The proposed therapy is to take myoblasts from normal donors and insert them into the muscles of DMD boys. The source of donor myoblasts would be from a histocompatibility-matched donor (often the father) where

problems of immune rejection would be reduced and where the presence of the normal counterpart of the dystrophin gene and its protein product would replace that missing in the DMD muscle. The object is to effect fusion of these cells with the muscle fibres of the DMD boys which are themselves dystrophin-negative. Fusion of dystrophin-positive donor myoblasts with dystrophin-negative DMD muscle fibres would thus, at least partially, restore dystrophin function to the fibres. Thus, E. Gussoni *et al.*, Nature *356*, 435-438 (1992) treated eight boys with DMD by transplanting normal myoblasts from a father or an unaffected sibling into their muscle. Three patients with deletions in the dystrophin gene expressed normal dystrophin transcripts in muscle biopsy specimens taken after myoblast injections. Using the polymerase chain reaction, these authors established that the dystrophin gene was expressing normal dystrophin in these biopsy specimens. Although in this work, the numbers of dystrophin-positive fibres produced in DMD muscle were low, the method holds some promise. D. J. Watt *et al.*, Nature *368*, 406-407 (March 1994) injected myoblasts carrying the *lac* Z gene encoding β-galactosidase (β-gal) into the muscle of first host *mdx* mice and subsequently grafted this muscle into second host *mdx* mice, surrounding the grafted muscle with "Spongostan" supported medium. When the neighbouring muscle in the second host mice was injured, the myoblasts containing the *lac* Z gene migrated through the Spongostan to produce β-gal-positive and dystrophin-positive myotubes adjacent to the injured muscle. By contrast, this effect did not normally occur when the muscle was uninjured. However, in one case of uninjured muscle spontaneous regeneration of the muscle, typical of *mdx* mice, was occurring and here the β-gal/dystrophin-positive fibres were seen in the surrounding muscle. These results show that implanted myoblasts migrate towards areas of muscle injury and muscle regeneration. This movement may be the result of some chemotactic influence exerted by injured muscle fibres.

A further advance on this type of therapy would be to use the DMD boy's own dystrophin-negative myogenic cells and insert the missing gene into them, which is easily achievable and the route contemplated for many forms of gene therapy on varied tissues. Recently, G. A. Cox *et al.*, Nature *364*, 725-728 (1993) demonstrated that the over-expression of dystrophin in transgenic *mdx* mice containing a full length cDNA encoding murine dystrophin appeared to prevent dystrophic symptoms and to have no

harmful side effects. However, although this therapy removes the problem of immune rejection of a "non-self" cell, it poses a problem not encountered with using donor cells of a non-self source. The myogenic cells of DMD boys are further down the senescence pathway than "normal" non-DMD myoblasts, for they have already passed through several bouts of degeneration/regeneration and hence mitoses. Given the Hayflick phenomenon, that cells divide a quantum number of times (50) before death, the strategy of using these "older" cells is not ideal.

In recent years, therefore, interest has centred on these two approaches as the most promising. Further prior art, of the context of which would not be clear without knowledge of the present invention, is referred to under "Summary of the invention".

It has now been found that an alternative approach may offer advantages, as it can avoid using cells which might not be well enough matched to prevent immune rejection and also avoid use of "older" cells.

SUMMARY OF THE INVENTION

The present invention is based on the radically different idea of using fibroblasts of dermal origin as the donor cells. Surprisingly, it has been found that when dermal fibroblasts are implanted in muscles of an animal which has a muscular disorder, these donor fibroblasts appear to fuse together to make a multinucleate cell which behaves like a muscle cell. Since these newly-formed cells express the same products (e.g. dystrophin, desmin, carbonic anyhdrase III) as are specific to native muscle cells in the normal animal (and, therefore, not found *inter alia* in the donor fibroblasts), they are considered to have been converted to the myogenic lineage.

Although it appears that muscle and lung fibroblasts as donor cells have some beneficial effect, it is small, whereas dermal fibroblasts give a large effect. An important aspect of the invention is therefore defined as a method of treatment of muscular disorder in a patient, which comprises administering to or adjacent to the muscle cells of the patient immunologically compatible dermal fibroblasts under conditions effective to convert the dermal fibroblasts to myogenic cells capable of producing products expressed by muscle-specific genes. In countries where a method of therapeutic treatment is not protectable, the use of the donor cells for the stated purpose or in the preparation of a formulation of the cells for the stated purpose should be substituted for the above method definition, as legally appropriate or conventional.

FURTHER PRIOR ART

In the work of J. E. Morgan *et al.* (1990) *supra*, the donor muscl cells implanted were a mixture of myogenic and non-myogenic cells, no steps being taken to separate the non-myogenic cells, which must have comprised fibroblasts. However, the authors refer to "cause for optimism with regard to the use of myogenic cells for treatment of inherited myopathies" and suggest investigation *inter alia* into whether myogenic cells obtained from different developmental stages of muscle differ in the efficiency with which they can restore or maintain normal muscle histology and function".

Ten years ago H. M. Blau *et al.*, Science *230*, 758-766 (1985), in a paper entitled "Plasticity of the Differentiated State", used muscle cells to determine the extent to which highly specialised cells were still capable of expressing genes of the nuclei of other cell types. Mouse muscle cells were fused *in vitro* with cells of other phenotypes. In these fused cells or heterokaryons, muscle gene expression was activated in the non-muscle nuclei of the fused cells. The non-muscle cells included four kinds of fibroblasts, one of which was skin fibroblasts. The authors concluded that this activation must be mediated by molecules transplanted to nuclei through the cytoplasm.

Six years ago, S. Chaudhari *et al.*, Nature *341*, 445-447 (1989) described the *in vitro* fusion of genetically defective (dysgenic) mouse myoblasts with fibroblast cell lines including *inter alia* rat dermal fibroblasts. Although dysgenic myoblasts can form myotubes they are unable to contract. The myotube is further along the differentiation pathway from the myoblast. The experiment involved adding the fibroblasts to myoblasts plated out from a culture. The authors remarked that fibroblasts seem to fuse very rarely if they are added after the myotubes have fully formed. They presume that the muscle cytoplasm induces the fibroblast nucleus to express muscle-specific genes, citing the Blau *et al.* paper. The authors add:

> "We do not yet know whether a similar fusion process can occur *in vivo* or, conversely, whether mechanisms exist to prevent such fusions. If fibroblasts can fuse with developing muscle *in vivo*, they might serv as a useful donor cell-type for the treatment of genetic diseases of muscle in humans".

Th authors do not xplain how such treatment would be helpful if the muscles are not developing, but wasting, nor do they indicate that fusi n wholly between fibroblasts might occur. Their work was concerned solely with *in vitro* fusion of fibroblasts to muscle cells.

The Chaudhari *et al.* work has been neglected by the art, presumably because, for the above reasons, there was no reason to suppose that conversion of the dermal to a myogenic lineage would occur *in vivo* and because other treatments involving muscle cells appeared more promising.

During the priority year, the invention was published in a paper by Gibson *et al.* (including the inventors) in Journal of Cell Science *108*, 207-214 (1995). The publishers have reported an unexpectedly heavy demand for reprints of the paper.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The treatment is based on the notion that at least some of the donor fibroblasts fuse with each other, express muscle-specific genes, and thereby become cells of the myogenic lineage, notably muscle cells or satellite cells thereof. The effect is most easily seen in the irradiated *mdx* mouse. When the same human fibroblasts as may be administered to a human patient, but possibly carrying a marker, are administered to irradiated *mdx* mice, the effect can be observed and such a test in mice may be considered as a test of the competence of the dermal cells (if any be needed) to be effective in the treatment of humans. Normally, the treatment aims to increase the number of dystrophin-positive cells found in the muscle of the treated patient, e.g. by muscle biopsy, the production of the muscle-specific protein dystrophin being regarded as a measure of success in conversion to normal muscle.

This invention has been particularly well demonstrated by Example 8, in which dermal fibroblast cells, from a human dystrophin gene-positive transgenic mouse, were implanted into an *mdx* mouse lacking dystrophin-positive muscle fibres. The *mdx* mouse muscle fibres became dystrophin positive and this gene could only have been provided by the donor cells. Thus, the gene was chromosomally incorporated into the dermal fibroblasts and expressed in a different cell type, viz. muscle cells.

The invention relates primarily to skeletal muscle but could be applied to cardiac or smooth muscle. It is of interest mainly in relation to muscular dystrophy, particularly DMD or BMD, but could be applied to any wasting or degenerative disorder of the muscle,

including injured or damaged muscle. This includes traumatic injury, but not, of course an injury so severe that the muscle "architecture" is wholly destroyed. Where the patient lacks or substantially lacks dystrophin-producing or another muscle-specific gene or possesses such a gene in a sub-normal concentration, the dermal fibroblasts administered contain the relevantly required DNA. The dermal cells may be transfected with this DNA in any of the usual ways, preferably so that it is incorporated within the cell nucleus. One such way is to incorporate the relevant DNA in a viral vector such as an adenovirus or adenovirus-associated vector and transfect the fibroblasts with the vector. Subject to safety requirements, a retroviral vector could alternatively be used. Dermal fibroblasts have been infected with retroviral vectors for gene therapy, see e.g. J. H. Axelrod *et al.*, Proc. Natl. Acad. Sci. USA, *87*, 5173-5177 (1990), D. St. Louis and I. M. Verma *ibid.*, *85*, 3150-3154 (1988), T.D. Palmer *et al.*, Blood *73*, 438-445 (1989) and R. I. Garver Jr. *et al.*, Science *237*, 762-764 (1987). Such methods could be used, substituting dystrophin or other muscle-specific DNA for the factor IX and alpha-1 anti-trypsin genes of these references. Another way is direct injection of the muscle-specific DNA into the fibroblasts. The manner of incorporation of the relevant DNA into the fibroblasts is not critical and other methods will be apparent to those skilled in the art. The relevant DNA may be a full length gene or only part thereof. A partial gene such as the so-called dystrophin minigene, see M. Dunckley *et al.*, FEBS *296*, 128-134 (1992) is often useful, for both BMD and DMD, especially where a retroviral vector is being used. The full length dystrophin gene, which has been described by N. Wells *et al.*, Human Mol. Gen. *1*, 35-40 (1992), is useful in methods where no vector can present or package DNA of such a long length. Appropriate regulatory sequence is included as may be required by the technique used, as is well known in the art.

Preferably the patient is not totally lacking in muscle cells when the treatment of the present invention is applied. A chemotactic factor present in the muscle cells may aid in the conversion of fibroblasts to muscle cells.

The invention includes specifically dermal fibroblasts containing, especially within the cell nucleus, a muscle-specific DNA, especially DNA encoding part or all of the dystrophin-protein. The DNA may be a full length or a partial gene, as noted above. Cell suspensions and biologically pure, especially serum-free, culture media containing such

cells are also included within the invention. Nothing in this specificati n is to be construed as laying claim to rights in a part of the human body except when isolated therefrom.

Where the muscle disorder results from injury and the patient has normal muscle-specific genes, the donor fibroblasts need not contain any foreign DNA.

The donor cells must be immunologically compatible with the patient to be treated in order to prevent their being rejected as foreign. Thus, some attempt will normally be made to match HLA type, as in other kinds of treatment involving the donation of body tissue. Preferably the donor cells are from the patient being treated. These cells are very easily harvested, e.g. by biopsy, to remove tissue from the dermis.

The route of administration of the donor cells is not critical so long as they are brought into intimate or near contact with the patient's muscle cells. Intramuscular injection (into the muscle) is the preferred route; intermuscular injection (between muscles) is possible. Other methods are implantation, to open up the skin and expose the muscle, and intra-arterial injection, e.g. into the femoral artery.

It has been found experimentally that dermal fibroblasts implanted into one muscle migrate to other, nearby muscles and there contribute to the formation of new muscle fibres and within these fibres express a muscle-specific gene.

The cells can be prepared for administration as follows. Skin biopsies are taken from the DMD patient to include the dermal layer. Dermal fibroblasts are grown in culture, but in serum-free medium, in case when the dermal cells are re-introduced to the patient, elements of animal serum proteins cause any immune response or contamination problems. Thus, a medium using serum-free supplements is normally required. Following growth of the dermal cells in culture, the normal counterpart of the defective gene (when required) is delivered to the cells. The cells are formulated as a suspension in a serum-free medium or as a cell pellet. Virological and bacteriological testing is compulsory before re-implantation.

The donor cells will normally be administered at a single time and at more than one site in the affected muscle. Generally, they will be administered, normally by injection, at 30 or more sites, especially 30-100. The total amount of dermal cells administered will usually be in the range 10^6 to 10^{10} for humans.

While the invention is mainly of interest for treating human patients, it includes

treatment of non-human animals such as pets, livestock and animals used in the production of food. The dosage of cells will need adjustment with size of animal, but this is well within the competence of those skilled in the art..

The following Examples illustrate the invention.

The words "POLYBRENE", "SPONGOSTAN", "FUNGIZONE" and "TRITON" are Registered Trade Marks.

EXAMPLE 1

1. Preparation of Dermal Fibroblasts

The skin from neonatal normal mice of strain C57B1/10ScSn was harvested by ventral incision along the length of the anterior abdominal wall and removing the skin from the underlying tissues. The skin was placed for 2 minutes in a petri-dish containing 70% alcohol to remove contaminants from the skin which would cause infection of cells in tissue culture. The skin was then removed to a petri-dish containing phosphate buffered saline (PBS) containing the antibiotics 80 IU/ml penicillin and 80 mg/ml streptomycin and 1% "Fungizone" (250 µg/ml amphotericin). Any adherent fat was scraped from the hypodermic surface of the skin.

Then the skin was placed in a petri dish containing Basal Salt Solution (BSS) containing penicillin and streptomycin (as above) and cut using crossed scalpel blades into 1 mm^3 pieces. The small pieces were removed singly into a tissue culture flask providing a 25 cm^2 growth area containing the minimum amount of growth medium. Minimum amount of medium (1 ml for the 25 cm^2 growth area TC flask) allows attachment of skin pieces to substratum. The growth medium was "DMEM" (Dulbecco's Minimal Eagle's Medium) supplemented with 2 mM L-glutamine, 88 IU/ml penicillin/88 mg/ml streptomycin (all supplied by Life Technologies Ltd) and 10% Fetal Calf Serum (Labtech International). The culture vessel was placed in a 37°C incubator in 5% CO_2. Three hours after transplanting the skin pieces an additional 1-2 ml of growth medium was added to ensure that the skin did not dry out.

The skin was left in culture until fibroblasts grew out from explanted skin. The medium was changed every 3 days during this time. When sufficient outgrowth had occurred, the skin pieces were removed to allow the culture to become sub-confluent.

On reaching sub-confluency, cells were washed in 1x phosphate buffered saline (PBS) and detached from the substratum using 0.25% Trypsin (Life Technologies). The action of trypsin was stopped after detachment of cells by the addition of growth medium containing 10% fetal calf serum. Cells were pelleted by centrifugation at 350 g for 10 minutes and resuspended in growth medium (as above) to a concentration of 2×10^5 cells/ml. This suspension was then plated into culture vessels and grown in the same growth medium as above until sub-confluency. This cycle of operations constituted a single passage.

The cells were passaged thus at least 6 times before implantation into mouse muscle. Some cells were ring-cloned, after passage 19, as follows.

Cells were trypsinised as above, and diluted in growth medium to 100 cells in 10 ml of growth medium. These were plated into 100 mm petri-dishes to induce single cells attachment to the substratum. When single cells were detected, cloning cylinders (2 mm internal diameter cut from "Perspex" tubing) were placed over such single cells to ensure such single cells grew as clones. Clones were harvested by trypsinization once each had produced a colony of 12-15 cells from the original one cell isolated.

2. Preparation of Muscle Fibroblasts

Muscle cells were prepared from muscle tissue of neonatal mice by disaggregation with trypsin and pangestin, exactly as detailed in D. J. Watt *et al.*, J. Neurological Science *57*, 319-331 (1982). The cells were plated out and left for 40 minutes in an incubator, during which time the fibroblastic cells adhered to the plate, but myogenic cells remained in suspension. Growth medium containing myogenic cells was removed and fresh growth medium added to the original flask with attached fibroblasts. Contaminating cells other than fibroblasts were still present in these cultures, but after passage at least 6 times the fibroblast became the predominant cell in the culture and such fibroblasts were used for injection. They were determined to be 100% fibroblastic using standard ultrastructural methods and immunocytochemistry. Immunocytochemistry was performed using commercially available anti-desmin antibodies (from Sigma Chemical Co.) which recognised intermediate filament proteins characteristic of myogenic cells. All cloned cells were desmin-negative, indicating they contained no myogenic cells. This was confirmed by their appearance at the ultrastructural level, which also indicated that they were indeed fibroblastic.

3. Preparation of Mice for Implantation of Dermal Fibroblasts

In all experiments nude *mdx* mice were used as recipients. The athymic nude mouse is immunologically compromised, possesses no thymus and hence will accept tissue from any other strain of mouse and indeed xenografted material without the fear of rejection of the implanted foreign tissue.

The right hind limb of each host x-linked muscular dystrophic (*mdx*) mouse was subject to 1,800 rads X-irradiation. This treatment knocks out the endogenous muscle precursor cells and renders the *mdx* muscle incapable of regeneration and hence more akin, histopathologically to DMD muscle. The method for such irradiation is as indicated in Wakeford *et al.*, Muscle & Nerve, *14*, 42-50 (1991).

The left lower hind limb of host mice remained unirradiated and hence will accept tissue from any other strain of mouse and indeed xenografted material without the fear of rejection of the implanted foreign tissue.

4. Injection of Fibroblasts into *Mdx* Recipients

Both dermal and muscle fibroblasts (in separate experiments) derived from the normal C57Bl/10ScSn strain of mouse, the normal strain from which the *mdx* mouse spontaneously arose, were injected into the tibialis anterior (TA) muscles (right irradiated and left unirradiated) of the host athymic nude *mdx* mouse. 3×10^5 cells were injected using a thin pipette with microlitre markings, flamed and pulled to a very fine point. The cells to be injected were spun into a pellet so that approximately 10 µl of cell suspension were injected into the very superficial fibres of the TA muscles, following the protocol developed in D. Watt *et al.*, Journal of Muscle Research and Cell Motility *14*, 121-132 (1993).

5. Analysis of Muscles

3 and 6 weeks after implantation, TA muscles were removed and frozen in melting isopentane cooled to -165°C by liquid nitrogen. Cryostat sections were cut until the site of injection had been reached, at which time 8 µm thick cryostat sections were cut. The first section was taken for histological examination, the second for dystrophin immunostaining and the third for glucose-6-phosphate isomerase (GPI) characterisation.

(a) Histological examination

Sections for histological examination were washed in distilled water for 2 minutes,

stained in haematoxylin for 2 minutes and washed in running water for 1 minute. After being differentiated in 70% alcohol containing 1% v/v hydrochloric acid for 5 seconds (to differentiate nuclei from cytoplasm) they were "blued" in running tap water for 5 minutes ("blueing" converts acid haematoxylin to neutral haematoxylin which gives the stain its characteristic blue colour). Sections were then counterstained in eosin for 1-2 minutes before being rinsed briefly in water and briefly dipped in 70% alcohol. They were then dehydrated in 2 changes of 100% alcohol for at least 2 minutes (each change) and immersed in 2 changes (2 minutes each) in xylene to clear them before being mounted in a xylene-based mountant.

This examination showed that the sections contained small and variable diameter fibres with non-peripherally placed nuclei characteristic of newly formed regenerated muscle fibres.

(b) Dystrophin immunostaining

Further sections were taken, some 100 μm and 200 μm deeper than the first, 8 μm sections, and the three sections from these different levels were immunocytochemically stained for dystrophin by incubating for one hour with a rabbit anti-dystrophin primary antibody, followed by a one hour incubation with anti-rabbit biotinylated IgG as secondary antibody and visualised using an avidin-fluorescein conjugate as label. Sections were counterstained for 20 minutes with 0.5 μg/ml propidium iodide. The majority of dystrophin-positive fibres were centrally nucleated.

Some anti-dystrophin antibodies are commercially available, but in this test the inventors used the P6 antibody from Dr. P. N. Strong, Dept. of Paediatrics and Neonatal Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0NN, U.K. : see T. G. Sherratt *et al.*, Biochem. J. *287*, 755-759 (1992). P6 recognises the -COOH terminus of dystrophin.

Immunofluorescence images were acquired using a Photonics Science Labstar intensified CCD camera attached to an Olympus BH2 fluorescence microscope. Following acquisition, images were stored on an optical disk. Quantitation of the area of the section that was immunofluorescently labelled was carried out and the territory occupied by fluorescent muscle fibres expressed as a volume fraction (percentage of the frame labelled). Results are shown in Table 1. *Mdx* mice are dystrophin-negative and in irradiated muscle

remain so. Dermal fibroblasts implanted into irradiated *mdx* muscle resulted in strikingly higher proportions of dystrophin-positive fibres as compared with non-irradiated muscle, when examined after 21 days. The lower percentage of dystrophin positivity at 42 days in irradiated muscle reflects the organisation and compactness of the positive fibres seen histologically. Lower proportions of dystrophin-positive fibres observed after implanting cloned dermal fibroblasts could be the result of using passage 19 cloned cells which may have lost some pluripotency. After implanting muscle fibroblasts (uncloned), higher levels of dystrophin-positive fibres were observed than would be expected from revertant fibres, suggesting some participation of implanted cells in muscle fibre formation.

TABLE 1

Source of implanted Cells	Days after Implantation	Vol. % sections showing dystrophin positivity	
		Irradiated	Non-irradiated
Dermal Fibroblasts uncloned	21	49	6
	21	55	1
	21	49	8
	42	17	2
	42	5	2
	42	4	2
Dermal Fibroblasts cloned	21	27	8
	21	20	2
	21	17	0.2
	21	14	2
Muscle Fibroblasts uncloned	21	1	2
	21	1	3
	21	3	4
	42	3	0.8
	42	2	1

(c) GPI characterisation

The isoenzymes of GPI were used to assess the relative contribution of host and donor cells to the muscle fibres present in the regenerating implanted TA muscles as follows:

The cryostat section was overlaid with a small piece of filter paper in order to absorb out the GPI isoenzymes. The filter paper with the GPI isoenzymes absorbed to it was loaded onto the surface of an agarose gel and electrofocused. Sites of GPI activity on the gel were revealed by using an agar overlay containing a reaction mixture of fructose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase, phenazine methosulphate and nitroblue tetrazolium.

Isoelectrofocusing separates three isoenzyme types of GPI in the mouse. The most anodally positioned isoenzyme consists of two sub-units coded for by the GPI-1b gene, and characterizes tissues derived from homozygous GPI-1b/GPI-1b mice, such as the 129/ReJ strains of the donor mice. The most cathodally positioned isoenzyme comprises two sub-

units coded for by the GPI-la gene, and characterizes homozygous GPI-la/GPI/la mice, such as the C57Bl/10ScSn strain of the host (recipient) mice. In muscle grafts, however, a "heterodimeric" isoenzyme form arises by association of one sub-unit coded for by the GPI-la gene, and one sub-unit coded for by the GPI-lb gene within the cytoplasm of the syncytial skeletal muscle fibre. Thus, a heterodimer occurs if mosaic muscle fibres have formed by the fusion of host and donor mono-nuclear precursor cells, resulting in the expression of both host and donor GPI genes with a common cytoplasm. For further detail of the method, see D. J. Watt *et al.*, Neuromuscular Disorders *1*, 345-355 (1991).

EXAMPLE 2

Expression of muscle specific genes within the muscle of the *mdx* mouse implanted with mouse dermal fibroblasts

The gene product chosen in this Example to show the expression of muscle-specific proteins within the *mdx* muscle implanted with mouse dermal fibroblasts was carbonic anydrase III which is expressed in early differentiating muscle. Four *mdx* nude mice were injected with 3×10^5 cloned mouse dermal fibroblasts, prepared as in Example 1, part 1, into both irradiated and non-irradiated *mdx* tibialis anterior muscle. To investigate the expression of carbonic anydrase III, standard immunocytochemical staining of cryostat sections cut from the muscles was carried out. The primary antibody was rabbit anti-carbonic anydrase III (CA III) [obtained from Dr. Gary Coulton, Dept. of Biochemistry, Charing Cross and Westminster Medical School, London W6 8RF], the secondary antibody mouse anti-rabbit biotinylated IgG. Visualisation required a third layer of an avidin-fluorescein conjugate. In all cases double immunocytochemical staining, i.e. both for dystrophin and CA III was carried out. The results showed that there was a co-localisation of dystrophin and CA III. That is, the newly formed muscle fibres which were dystrophin-positive also expressed CA III, i.e. the same fibres expressed both of these muscle-specific gene products.

EXAMPLE 3

Conversion of dermal fibroblasts to a myogenic lineage in muscle regenerating after injury

This experiment was set up to investigate the effects of implanting dermal fibroblasts into muscle regenerating after injury and not due to disease, (e.g. *mdx*) muscle i.e., of normal C57Bl/10 mice. The fluorescent marker PHK-26 from Sigma Chemical Co. was used. This substance is a phospholipid which plugs itself into the cell membrane and remains there. It is said to remain in the membrane of the cells which are labelled for up to 100 days following the implantation of the cells *in vivo*. Cloned dermal fibroblasts were incubated *in vitro* with PHK-26, during which time the cell membrane was stably labelled with this compound. Such cells were then injected into the TA of normal C57Bl/10 mice as previously described and left for 3 and 6 weeks. Muscles were removed and frozen. Sections were cut from the regenerating muscle. Assay for dystrophin was not undertaken, as the host mice are dystrophin-positive. After 24 days the fluorescent marker was seen associated with the muscle cell membrane, suggesting that these cells arose from dermal fibroblasts..

In addition to using the PHK-26 marker, dermal fibroblasts carrying the *lac* Z gene coding for β-galactosidase were used for implantation into regenerating tibialis anterior muscles of normal (C57B1/10) mice.

Two sources of *lac* Z labelled cells were implanted:

(a) C57Bl/10 dermal fibroblasts harvested from skin of neonatal mice were infected with the mouse moloney leukemia virus retroviral (MoMLV) vector carrying the *lac* Z gene.

(b) Dermal fibroblasts were harvested from C57Bl "Rosa" mice, a *lac* Z transgenic mouse. All cells from this animal constitutively express β-galactosidase. Dermal fibroblasts from the "Rosa" transgenic mice were received from Dr. M. Skynner, Babraham Institute, Cambridge, U.K.

Results from these experiments show the presence of β-galactosidase positive fibres in the regenerated graft 21 days after implantation. These results indicate that dermal fibroblasts have participated in the formation of newly formed muscle fibres.

EXAMPLE 4

Further analysis of muscles

Example 1, part 5 was repeated on 23 *mdx* nude mice except that the third 8 μm section was taken for additional dystrophin immunostaining, instead of GPI analysis. The 8 μm sections and the further sections taken at 100 μm and 200 μm deeper levels were all stained as described, separately, with the P6 antibody and with another rabbit anti-dystrophin antibody H12, available from the same source. Whereas P6 recognises the carboxy terminus, H12 recognises the rod domain of the protein. The avidin-fluorescein conjugate of Example 1 was replaced by a streptavidin-"Texas Red" conjugate for P6 and by a streptavidin-fluorescein conjugate for H12.

Up to 80% of individual muscle sections were positive when stained with P6 and H12. All sections positive for P6 were also positive for H12, as result which shows that at least the majority of the dystrophin molecule is expressed.

EXAMPLE 5

Conversion of human dermal fibroblasts to newly formed muscle fibres in th *mdx* mouse muscle

An important step in using dermal fibroblasts as a route of therapy is to investigate whether human dermal fibroblasts also form dystrophin-positive fibres in *mdx* muscle. To this end, human dermal fibroblasts were injected into *mdx* mouse TA muscles using a similar protocol to that of Example 1.

The human dermal fibroblasts were derived from 3 sources: (a) from the facial skin of a 46 year old woman; (b) from the forearm of a 9 year old boy; and (c) from commercially available cells of neonatal foreskin.

At biopsy (under local anaesthesia) the skin sample from the 46 year old donor was placed in 10 ml of DMEM supplemented as in Example 1 except that Australian Fetal Calf Serum (Sigma Chemical Co.) was used. The skin was transferred to a sterile petri dish and then cut using crossed scalpel blades into 1 mm^3 pieces. The small pieces were removed singly into tissue culture flasks, each providing a 25 cm^2 growth area and each containing 1 ml of the above growth medium. The flasks were incubated as in Example 1, except that they were inverted to encourage adhesion of the skin pieces t the substratum of the flask

(hence skin pieces were made to hang on to the base of the flask which was placed upside down in the incubator). Additional growth medium was added as in Example 1 to ensure that the skin did not dry out.

The procedure then continued exactly as in Example 1, part 1.

The dermal cells from the 9 year old boy were received in a condition in which they had already been grown out from the skin.

The preparation of the mice and injection of fibroblasts into the mice were as described in Example 1, parts 3 and 4.

Some of the sections were then stained with anti-human slow myosin heavy chain (HuSMyHC) antibody. The slow myosin heavy chain is a human muscle-specific gene product. 8 μm sections were cut at 3, 4 or 5 different step levels through the muscle (varied from experiment to experiment). The cut sections were treated as follows:

1. The sections were air-dried for 15 minutes.
2. The sections were blocked in 10% Fetal Calf Serum. 10% Horse Serum, 10% Goat Serum for 30 minutes. This step is used to prevent non-specific binding of antibody to any vacant sites on the muscle cell surface.
3. The sections were washed for 5 minutes in Phosphate Buffered Saline (PBS), followed by a further 15 minutes in PBS containing 0.05% "Triton X-100".
4. The sections were incubated at room temperature in anti-HuSMyHC antibody, by diluting the commercially available antibody 1:2 in 0.05% "Triton X-100" for 1 hour.
5. The sections were washed in staining troughs 3 times for 5 minutes each, followed by a final 20 minute wash, all in PBS.
6. The sections were incubated at room temperature in goat anti-mouse biotinylated IgG 1:100 dilution in PBS, using 50 μl per section.
7. Washing step 5 was repeated.
8. The sections were incubated in the dark in streptavidin/fluorescein for 30 minutes, using a 1:100 dilution in PBS.
9. The sections were washed twice for 10 minutes and then for a further 20 minutes in PBS.
10. They were mounted in "Entellan" mounting medium and stored at 4°C before viewing under a fluorescent microscope.

Others of the sections were immunostained with the P6 and H12 antibodies as described in Example 4. Using all three sources f cells, it was found that implantation into irradiated muscles of the *mdx* mouse resulted in the presence of small calibre, newly-formed dystrophin-positive fibres. Fewer were seen than when mouse cells were implanted into mouse tissue, but the numbers were still far greater than expected to be due to mouse revertant fibres. The best results were obtained using the neonatal foreskin cells as the donor cell for implantation. In muscle sections right in the centre of the muscle and near the injection site as many as 68 dystrophin-positive fibres were found, arranged in two groups - one of 33 fibres and the other of 35 fibres flanking the injection site. Greater numbers of dystrophin-positive fibres were found than have been reported by other workers following the injection of human myogenic cells into nude mouse muscles. The best result so far is the presence of 5% of dystrophin-positive fibres following implantation of 3×10^5 cells into the mouse muscle. Other researchers have implanted significantly higher numbers of myogenic cells into the TA muscle of nude mice. These hosts were not *mdx* but they used a dystrophin antibody which is specific for human dystrophin to show that the newly-formed dystrophin-positive fibres were formed by the fusion of host myogenic cells with mouse muscle. Using nude host mice, their best result was 3.6% of dystrophin-positive fibres and this was after the implantation of 10^7 cells, i.e. 33 times more cells than were injected here.

EXAMPLE 6

Introduction of a gene into mouse dermal fibroblasts, implantation of the fibroblasts into the *mdx* mouse and expression of the gene in muscle fibres of the host mouse

Mouse dermal fibroblasts for implantation into *mdx* mouse muscle were infected with the Mouse Moloney Leukemia Virus Retroviral Vector carrying the *lac* Z gene. This gene codes for β-galactosidase, which when expressed within cells stains a blue colour when the usual substrate for β-galactosidase is used in the staining procedure.

The producer cells containing the retroviral vector were obtained from Dr. J. Price, National Institute for Medical Research, Mill Hill, London but this vector is widely available. In addition to the retrovirus carrying the lacZ gene it also contains the neomycin-resistance gene. Thus when target cells are infected with the retroviral v ctor any cell

which becomes infected will also carry the neomycin-resistance gene [Price *et al.*, Proc. Natl. Acad. Sci. USA *84*, 156-160 (1987)]. This allows selection of the cells which have become infected by the retrovirus and hence carry the 2 inserted genes - for LacZ and neomycin resistance. Selection is carried out by growing cells in growth medium not supplemented with penicillin and streptomycin as previously described, but supplemented with 400 μg/ml of Geneticin, a neomycin analogue (Sigma Chemical Co). Only the cells which have become infected with the retroviral vector will survive in this selection medium.

Mouse dermal fibroblasts were prepared as in Example 1, part 1 to sub-confluency. The culture medium was removed and replaced with 3 ml of medium which had been used to sustain the growth of the producer cell line which packages the retroviral vector. To the culture medium was added 8 mg/ml of "Polybrene", a reagent that helps the retrovirus to attach to the target cells, i.e. in this case dermal fibroblasts. The producer-cell medium was removed after 3 hours and replaced with normal growth medium. After 24 hours the growth medium was removed and replaced with medium containing 400 μg/ml of the neomycin Geneticin. Within 24 hours of infection of cultures with the retroviral vector, those cells which have not been retrovirally infected stop dividing and by one week they die. The remaining cells were therefore kept in the medium containing Geneticin and grown to increase their numbers. All these cells that survived contained the *lac* Z gene.

The dermal fibroblasts, infected with the retrovirus carrying the β-galactosidase gene, were implanted into the muscle of *mdx* nude mice. Three weeks after this implantation, the muscles were removed and prepared for sectioning as described in Example 1 section 5, but in this case sections were histologically examined and were also analysed for β-galactosidase activity. This involved cutting 12 μm cryostat sections (as opposed to 8 μm for histological and dystrophin analysis reported in Example 1). Sections were treated as follows:

1. Fixed in 0.5% glutaraldehyde in PBS for 15 minutes on ice.
2. Washed briefly in PBS + 2mM magnesium chloride, then for 10 minutes in fresh wash solution (PBS + 2mM $MgCl_2$), on ice.
3. 10 mins in detergent solution on ice (i.e. PBS + 2mM $MgCl_2$, 0.01% sodium desoxycholate, 0.02% "Nonidet P40").

4. Incubated overnight in the dark at 37°C in X-gal solution, the substrate for β-galactosidase, i.e. 20mM potassium ferricyanide; 20mM potassium ferrocyanide; 2mM $MgCl_2$, 0.01% sodium desoxycholate, 0.02% "Nonidet P40", 1mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside).
5. Washed 2 x 5 mins in PBS at room temperature.
6. Washed once, briefly, in distilled water.
7. Dehydrated through alcohol, i.e. 5 mins 50%; 5 mins 70%; 5 mins 100%.
8. Treated 2 x 5 mins with "Histoclear" (clearing agent).
9. Mounted in DPX.

The patches of blue colour obtained on the X-gal plates indicate that the *lac* Z gene was successfully expressed in the mouse muscle.

Also, these same muscle fibres were found to be dystrophin-positive by immunostaining: again, there was localisation of β-galactosidase production with dystrophin staining, using both the P6 and H12 antibodies (see Example 4). These results therefore indicated that a gene introduced into the dermal fibroblast *ex vivo* can be expressed within muscle fibres of the irradiated *mdx* mouse when implanted into the mouse and that these fibres are also dystrophin-positive. This is another significant result, for it shows that these fibres are definitely not revertant fibres as they express the inserted *lac* Z gene and also the dystrophin gene product which is not normally present in the *mdx* mouse.

EXAMPLE 7

Introduction of a gene into mouse dermal fibroblasts, implantation of the fibroblasts into normal mice having an implant of injured muscle tissue and expression of the gene in regenerating muscle fibres

Experiments have also been carried out to determine whether the *lac* Z gene would be expressed in muscles regenerating after traumatic injury. The procedure was similar to the above except that mice of the C57B1/10 strain were used. These mice express the dystrophin gene, but the introduction of the *lac* Z gene into the dermal fibroblasts enables the fibroblast origin of newly-formed muscle fibres to be verified.

An initial experiment was carried out in which the irradiated TA muscle of the host mouse was minced and re-inserted in minced state into the host leg. When 3×10^5 *lac* Z-

containing mouse dermal fibroblasts were implanted into the minced muscle in these mice, no regeneration of muscle was seen, the muscle site being replaced by fibrofatty connective tissue. Also, no blue coloration was observed.

It was felt that the above experiment was unrealistically severe, as the normal architecture of the muscle was completely disrupted by the mincing. This work was therefore repeated, but using whole muscle grafts. This involved cutting the distal tendon of the host's extensor digitorum longis (EDL) muscle and suturing it back in place. This causes the muscle to totally degenerate and regenerate because it is cut off from its nerve and blood supply during the procedure of distal tendon cut. It then regenerates and its nerve and blood supply re-enters the muscle during the regeneration stage. The suture of the EDL was tied around the distal tendons of the adjacent peroneal muscles to ensure that the EDL muscle was held under tension - as described in Watt *et al.*, Nature *368*, 406-407 (1994) [or, more comprehensively, D.J.Watt *et al.*, J. Mus. Res. Cell. Motil. *14*, 121-132 (1993)]. [If the muscle is not held under tension, the muscle atrophies and the experiment fails]. Three days after the tendon was sutured the dermal fibroblasts were introduced into the belly of the muscle by injection using a thin pipette, as for Example 1, part 4. When 3×10^5 *lac* Z-containing mouse dermal fibroblasts were implanted into the EDL muscle regenerating in this way, a substantial number of β-galactosidase positive fibres were found when working with non-irradiated EDL (and with irradiated EDL, what then?).

EXAMPLE 8

Introduction into *mdx* mouse muscle of dermal fibroblasts derived from a transgenic mouse, carrying the human dystrophin gene.

This experiment was performed to show whether a dystrophin-negative mouse could be made to express the human dystrophin gene when this gene was introduced by implantation.

Dermal fibroblasts were derived from skin of the *mdx* mice which were transgenic for the full length human dystrophin gene i.e. they had the *mdx* background, but were dystrophin-positive because of the transgene - N. Wells *et al.*, Human Mol Gen. *1*, 35-40 (1992). The dermal fibroblasts from the *mdx* transgenic mouse were grown as in Example 1, part 1 and injected into irradiated *mdx* muscle as in Example 1, part 4. Three

weeks after implantation, the injected muscles were removed and cryostat sections cut from the muscles and analysed for histology and for the presence of dystrophin-positive fibres. On histological examination, sections were characterised by the presence of high numbers of immature, newly formed muscle fibres. When stained with P6 antibody, up to 40% of the area of individual muscle sections contained dystrophin-positive fibres. Thus in the *mdx* mouse implanted with dermal fibroblasts where the dystrophin gene had been introduced by a transgenic route, high numbers of dystrophin-positive fibres were observed. The sole source of dystrophin in these experiments was from the implanted transgenic dermal fibroblasts carrying the human dystrophin gene. The P6 anti-dystrophin antibody reacts with human or mouse dystrophin. The sections will therefore be stained with an antibody which is specific for human dystrophin.

CLAIMS

1. A method of treatment of muscular disorder in a patient, which comprises administering to or adjacent to the muscle cells of the patient immunologically compatible dermal fibroblast cells, under conditions effective to convert the dermal fibroblast cells to myogenic cells capable of producing products expressed by muscle-specific genes.

2. A method according to claim 1, wherein, when the dermal fibroblast cells are administered to mice under as nearly as possible the same conditions as in a non-mouse patient, at least a proportion of these donor dermal fibroblast cells are converted to myogenic cells by fusion between said donor cells.

3. A method according to claim 1 or 2, wherein the donor dermal fibroblast cells are taken from the patient to whom they are administered.

4. A method according to claim 1, 2 or 3, wherein the administration to the patient is by implanting the dermal fibroblast cells within or injecting them into or adjacent to the muscle of the patient.

5. A method according to claim 1, 2, 3 or 4, wherein the conversion is effective to increase the number of dystrophin-positive cells found in a biopsy of the muscle of the treated patient.

6. A method according to any preceding claim, wherein the dermal fibroblast cells administered contain a muscle-specific DNA which is not present in a normal concentration in the patient's muscle cells.

7. A method according to claim 6, wherein the patient is suffering from muscular dystrophy and the dermal cells administered contain the dystrophin gene or a part thereof.

8. A method according to claim 7, wherein the muscular dystrophy is Duchenne's.

9. A method according to any of claims 1-5, wherein the patient is suffering from an injury resulting in muscular degeneration.

10. Use of dermal fibroblast cells, for the preparation of a cell formulation for administration to an immunologically compatible patient under conditions effective to convert the fibroblast cells to myogenic cells capable of producing products expressed by muscle-specific genes.

11. Use according to claim 10 which further comprises any one or more of the features of claims 2-9.

12. Dermal fibroblast cells which contain muscle-specific DNA defined in claim 6 or 7.

INTERNATIONAL SEARCH REPORT

Inter[illegible]plication No
PC[illegible]/GB 95/02187

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/10 A61K48/00 A61K35/36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 329, no. 13, 1993 BOSTON, pages 915-920, SARA SANCHO M.D. ET AL 'Analysis of dystrophin expression after activation of myogenesis in amniocytes, chorionic-villus cells and fibroblasts' see the whole document, especially page 918, column 2, last paragraph --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 1995	05. 01. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fernandez y Branas,F

INTERNATIONAL SEARCH REPORT

International Application No
PC1/GB 95/02187

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, vol. 341, 1989 LONDON GB, pages 445-447, NIRUPA CHAUDHARI ET AL 'Restoration of normal function in genetically defective myotubes by spontaneous fusion with fibroblasts' cited in the application see the whole document	1-5, 10-12
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 192, no. 1, 1993 ORLANDO, FL US, pages 69-74, HUGNOT, J.-P. ET AL 'Expression of the dystrophin gene in cultured fibroblasts' see the whole document	12
X	HUMAN MOLECULAR GENETICS, vol. 1, no. 1, 1992 OXFORD, pages 35-40, WELLS, D.J. ET AL 'Human dystrophin expression corrects the myopathic phenotype in transgenic mdx mice' cited in the application see the whole document	12
X,P	JOURNAL OF CELL SCIENCE, vol. 108, no. 1, 1995 CAMBRIDGE, pages 207-214, GIBSON, A.J. ET AL 'Dermal fibroblasts convert to a myogenic lineage in mdx mouse muscle' cited in the application see the whole document	1-12
A	THE JOURNAL OF CELL BIOLOGY, vol. 111, no. 6, 1990 NEW YORK, pages 2437-2449, MORGAN J.E. ET AL 'Normal myogenic cells from newborn mice restore normal histology to degenerating muscles of the mdx mouse' cited in the application see the whole document	1-12
A	WO,A,91 12329 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 1991 see the wh le document	1-12
A	WO,A,89 02468 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 1989 see the whol document	1-12
A	US,A,5 219 740 (A.DUSTY MILLER) 1993 see the whole document	1-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB95/02187

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-9
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-9 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 95/02187

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9112329	22-08-91	AU-B-	7312891	03-09-91
		US-A-	5466676	14-11-95
WO-A-8902468	23-03-89	AT-T-	117375	15-02-95
		DE-D-	3852823	02-03-95
		DE-T-	3852823	24-05-95
		EP-A-	0378576	25-07-90
		EP-A-	0633318	11-01-95
		JP-T-	3500124	17-01-91
		US-A-	5460959	24-10-95
US-A-5219740	15-06-93	NONE		